Complete, long-lasting protection against malaria of mice primed and boosted with two distinct viral vectors expressing the same plasmodial antigen

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We report that complete protection against malaria and total inhibition of liver stage development and parasitemia was obtained in 100% of BALB/c mice primed with a replication-defective recombinant adenovirus expressing the circumsporozoite (CS) protein of *Plasmodium yoelii* (AdPyCS), followed by a booster with an attenuated recombinant vaccinia virus, expressing the same malaria antigen, VacPyCS. We found increased levels of activated CS-specific $CD8^+$ and $CD4^+$ T cells, higher anti-sporozoite antibody titers, and greater protection in these mice, when the time between priming and boosting with these two viral vectors was extended from 2 to 8 or more weeks. Most importantly, by using this immunization regimen, the protection of the immunized mice was found to be long-lasting, namely complete resistance to infection of all animals 3 1/2 months after priming. These results indicate that immunization with AdPyCS generates highly effective memory T and B cells that can be recalled long after priming by boosting with VacPyCS.

The most effective vaccination protocol against malaria, in animals, and also in humans, consists of the repeated immunization with radiation-attenuated sporozoites of malaria parasites. This immunization not only induces sporozoite-neutralizing antibodies, but also elicits effective cell-mediated immunity (1). Administration of this vaccine completely inhibits the development of mature liver-stages (exo-erythrocytic forms) of malaria parasites, avoiding the subsequent infection of red blood cells, responsible for all symptoms and pathology of the disease (2). However, the lack of an *in vitro* culture system to obtain and expand sporozoites prevents the widespread use of this vaccine. Furthermore, a large number of irradiated sporozoites are required to elicit protection in humans. The alternative, namely, the use of subunit vaccines, based on recombinant DNA technology or synthetic peptides, is currently making it possible to obtain amounts of selected malaria antigens, which are adequate at least for experimental use and limited vaccine trials in humans. *Plasmodium yoelii* is a highly infective malaria parasite, because inoculation of 10 or fewer sporozoites can result in patent infection. The infectivity of these sporozoites is comparable to that of *P. falciparum*, the most virulent species of malaria parasites infecting humans. Thus, protection against *P. yoelii* is, in our view, more meaningful than that elicited by other rodent malaria species, such as *P. berghei*, which requires between 10^2-10^3 sporozoites to produce infection (3).

The circumsporozoite (CS) protein is the most abundant antigen on the surface of malaria sporozoites, the initial mosquito-transmitted infective stage of parasites for vertebrate hosts (4). We had earlier shown that a single immunizing dose of a recombinant adenovirus, genetically engineered to express the CS protein AdPyCS, elicits complete resistance against *P. yoelii* infection in ≈40% of the mice (5). Thus, AdPyCS is certainly among the most effective viral vectors for priming the immune response. However, as with most viral vectors, repeated immunizations, at short time intervals, with AdPyCS failed to improve significantly the immune response resulting from one single administration.

Our earlier reports, subsequently confirmed by others, had shown that, independently of the source and mode of its presentation, the CS antigen elicits an immune response against malaria sporozoites. This response can be elicited by sporozoites, recombinant influenza viruses, plasmid DNA, recombinant toxoplasma, or Ty-VLP particles (2, 6–9). However, this response, elicited by recombinants or synthetic peptides, can be very modest. It can consistently be greatly enhanced by a booster with a recombinant vaccinia virus expressing the same antigen (VacPyCS). It was, therefore, of interest to determine whether, and under what conditions, immunization with a vector that elicited a very effective strong primary immune response, like AdPyCS, would be optimally enhanced by the booster with VacPyCS.

In the present report, we compared the immunogenicity in mice elicited by immunization with either AdPyCS alone, or AdPyCS, later boosted with VacPyCS. The corresponding CS-specific immune responses in these different groups of mice were evaluated for their capacity to elicit anti-sporozoite antibodies, specific T cell responses, and most important, to confer protection against challenge with viable sporozoites of *P.yoelii*. Our findings also provide some insight into conditions, optimal for generating memory T and B cells in this system, because both cell types are essential for developing an effective long-lasting vaccine.

Materials and Methods

Animals and Parasites. Six- to eight-week-old female BALB/c mice, purchased from the National Cancer Institute (Bethesda, MD), were used for all of the experiments. *P. yoelii* (17XNL strain) was maintained by alternate cyclic passages in *Anopheles stephensi* mosquitoes and Swiss–Webster mice, as described (10). Sporozoites obtained from dissected salivary glands of infected mosquitoes 2 weeks after their infective blood meal were used for challenge of the immunized and control mice. This challenge consisted of the injection of 50,000 viable sporozoites into the tail vein of mice 2 or more weeks after their immunization. The outcome of the challenge was determined by measuring the parasite burden in the liver of the mice by using a quantitative real-time PCR method. We also determined, in selected groups of mice, whether or not they developed parasitemia after i.v. inoculation of 75–100 sporozoites, by microscopic examination of Giemsa-stained thin blood smears obtained daily, from the third day after challenge up to day 14.

Recombinant Viruses and Immunizations. AdPyCS is a replication-deficient human type 5 recombinant adenovirus, expressing the

Abbreviations: reAd, recombinant adenovirus; CS, circumsporozoite; ELISPOT, enzyme-linked immunospot; IFA, indirect immunofluorescence assay.

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CS protein of *P. yoelii* under the regulation of the cytomegalovirus enhancer and promoter (5). VacPyCS is an attenuated recombinant vaccinia virus, expressing the CS protein of *P. yoelii* in the TK region of the viral genome and under the regulation of the virus early-late promoter P 7.5, generated as previously described (6, 11). Groups of mice were immunized s.c. with only AdPyCS. Their immune responses were monitored for 18 weeks and followed by challenge at various time points. To enhance this immune response, some of the mice were given a booster with 10^7 pfu/mouse of VacPyCS administered i.p. at various time points after priming and later challenged with sporozoites.

Detection and Quantification of *P. yoelii* Ribosomal RNA Sequences by Reverse Transcription (RT) and Real-Time PCR. Total RNA (2 μg), from the livers of mice inoculated with 50,000 viable sporozoites $\approx$40 h earlier, was reverse-transcribed, by using a previously described protocol (12). An aliquot of the resulting cDNA (133 ng) was used for real-time RT-PCR amplification of *P. yoelii* 18S rRNA sequences. This amplification was performed in a GeneAmp 5700 Sequence Detection System (PE Applied Biosystems). For this purpose, we used primers 5′-GGGGATTGGTTTTGACGTTTTTGCG-3′ (54 nM) and 5′-AAGCATTAAATAA AGCGAATACATCCTTAT-3′ (60 nM) together with the dsDNA-specific dye SYBR Green I, incorporated into the PCR reaction buffer (PE Applied Biosystems) to detect the PCR product generated. The temperature profile of the reaction was 95°C for 10 min, and 35 cycles of denaturation at 95°C for 15 seconds, and annealing/extension at 60°C for 1 min. Experiments performed by using this methodology showed that it accurately detects hepatic parasite burdens resulting from the inoculations of 3-fold different amounts of sporozoites ranging from 25, 75, 225 and so on, up to more than 164,000 sporozoites.

Quantification of Epitope-Specific $CD8^+$ and $CD4^+$ T Cells by the Enzyme-Linked Immunospot (ELISPOT) Assay. The relative number of CS-specific, IFN-γ-secreting, $CD8^+$ and $CD4^+$ T cells, in the spleens of mice immunized by using the different regimens, was determined by direct *ex-vivo* ELISPOT assays, as previously described (13, 14). For these assays, we used MHC-compatible A20.2J target cells coated with the peptides SYVPSAEQI (10) or YNRNIVNRLLGDALNGKPEEK (15), which contain the CS-derived H-2K^d-restricted epitopes recognized by CS-specific $CD8^+$ or $CD4^+$ T cells, respectively. Lymphocytes were incubated with APCs for 24 h before the assay was developed.

Indirect Immunofluorescence Assay (IFA). Sera from immunized mice were obtained at the time points indicated in each figure, and their anti-sporozoite antibody titers were determined by using *P. yoelii* sporozoites air-dried onto multispot glass slides. After 1 h of incubation of these antigen slides, with the different sera diluted in PBS containing 1% BSA, the slides were washed and incubated with an FITC-labeled goat anti-mouse Ig antibody for another hour (Kirkegaard & Perry Laboratories). After repeated washes, the slides were mounted, and the anti-sporozoite antibody titers were determined as the highest serum dilution producing fluorescence when viewed under a UV microscope.

Flow Cytometric Analyses. Fluorescence-activated cell sorter (FACS) analyses were performed by using a Becton Dickinson flow cytometer (FACScalibur). Detection of CS-specific lymphocytes was performed by staining these cells with a fluorescent H2-K^d tetrameric complex containing the CS-specific CD8 epitope SYVPSAEQI of P. *yoelii* inside their peptide binding groove. Further characterization of these lymphocytes was performed by combining this tetrameric complex staining with the several monoclonal antibodies obtained from PharMingen, i.e., anti-CD8a-APC (clone 53-6.7), anti-CD4-APC (clone GK1.5), and anti-CD62L-FITC (clone MEL 14). The percentage of apoptosis was determined in the CS-specific/$CD8^+$ lymphocyte population by end labeling the genomic DNA breaks with fluorescent dUTP nucleotides (*In situ* Cell Death detection kit, Roche Biochemicals).

Results

Level and Persistence of the Immune Response in Mice Elicited by Administration of a Single Dose of AdPyCS. In earlier experiments, we determined that, 2 weeks after being given a single s.c. administration of AdPyCS (10^9 pfu/mouse), mice acquired an appreciable degree of protective immunity against *P. yoelii* infection, documented by the sharp reduction of parasite rRNA in the liver, as well as sterile protection in $\approx$40% of these mice (5).

Our previous attempts to increase this immune response by administration of VacPyCS as a booster 2 weeks after priming failed to do so, resulting in only a modest increase. One possible explanation was that the strong immune response elicited by the large dose of AdPyCS, then used for priming, inhibited the efficacy of the VacPyCS booster. We, therefore, modified the prime/boost protocol in two ways. First, by reducing the priming dose of AdPyCS 10-fold, from 10^9 to 10^8 pfu/mouse. In doing so, the corresponding immune responses should diminish considerably. Next, we increased the time between priming and boosting, to allow the primary immune response to fully develop into a memory type response.

To determine precisely the characteristics and duration of the immune response elicited by a single s.c. dose of 10^8 pfu AdPyCS, we immunized BALB/c mice at different time points. Their spleen cells were obtained at 2, 4, 8, 12, or 18 weeks after immunization to compare the levels of CS-specific IFN-γ-producing $CD4^+$ and $CD8^+$ T cells. At the same time, anti-sporozoite IFA titers were measured in the sera of the different groups of mice (Fig. 1*a*). At each of these time points, additional mice were immunized and challenged with *P. yoelii* sporozoites. The levels of parasite 18S rRNA in the livers of the immunized mice were determined by using a highly sensitive real-time PCR (Fig. 1*b*). Finally, some of these animals were challenged with a smaller dose of sporozoites, 2 weeks after administration of AdPyCS, to determine whether they had developed sterile immunity (see controls in Table 1).

By 8 weeks after immunization, the levels of splenic CS-specific $CD8^+$ and $CD4^+$ T cells had decreased very appreciably from those detected at 2 weeks. At later times, the number of CS specific T cells remained constant, at this low level, for the following 10 weeks (Fig. 1*a*). Anti-sporozoite IFA antibody titers peaked 2 and 4 weeks after immunization, decreasing slightly between 4 and 8 weeks postimmunization and remaining constant for the next 10 weeks. These data on cell-mediated immunity strongly suggest that, by 8 weeks after immunization, a stable pool of memory T and, most likely, also memory B cells had been generated.

The levels of parasite inhibition (assessed by the number of copies of *P. yoelii* 18S rRNA) in the livers of AdPyCS-immunized mice injected 40 h earlier with viable sporozoites, were also determined at Weeks 2, 4, 8, 12, and 18 after priming (Fig. 1*b*). However, the maximum level of inhibition of parasite development in the liver of these mice, observed 2 weeks after immunization, was not sufficient to induce complete protection in identically immunized mice (see controls in Table 1).

Potentiation of the Anti-Malaria Immune Responses of Mice Primed with AdPyCS and Boosted with a Recombinant Poxvirus Expressing the Same "Foreign" Antigen. To greatly increase the anti-malaria immune responses and, in particular, to protect them from infection by sporozoite challenge, we boosted AdPyCS-primed mice with VacPyCS. This regimen was based on the results of our earlier research, which showed that, whatever the antigen used

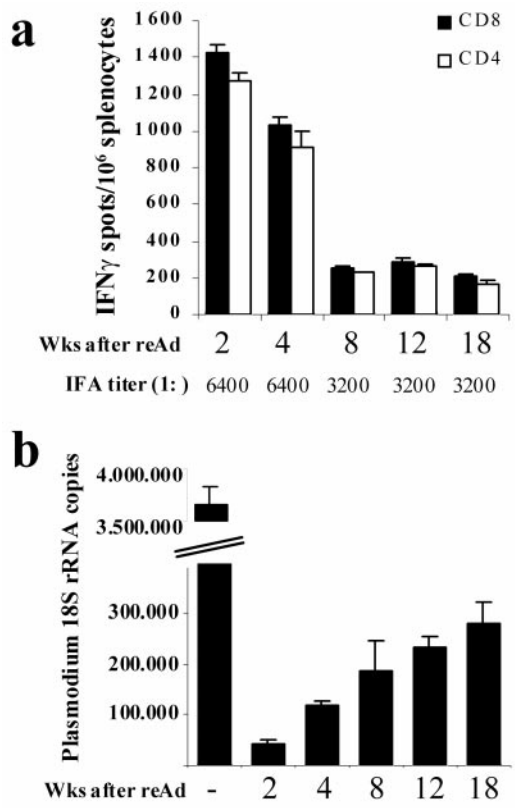

Fig. 1. Time course (in weeks) of immune responses and inhibition of liver stages in mice (groups of three) immunized with 10^8 pfu of reAd (AdPyCS). (*a*) Number of CS-specific $CD8^+$ and $CD4^+$, IFN-γ-producing T cells and corresponding antibody titers (IFA). (*b*) Inhibition of liver stage development expressed as number of copies of *Plasmodium* 18S rRNA. Mice immunized and challenged at the same time points as in *a*. Data in *a* and *b* represent the mean of two identical experiments.

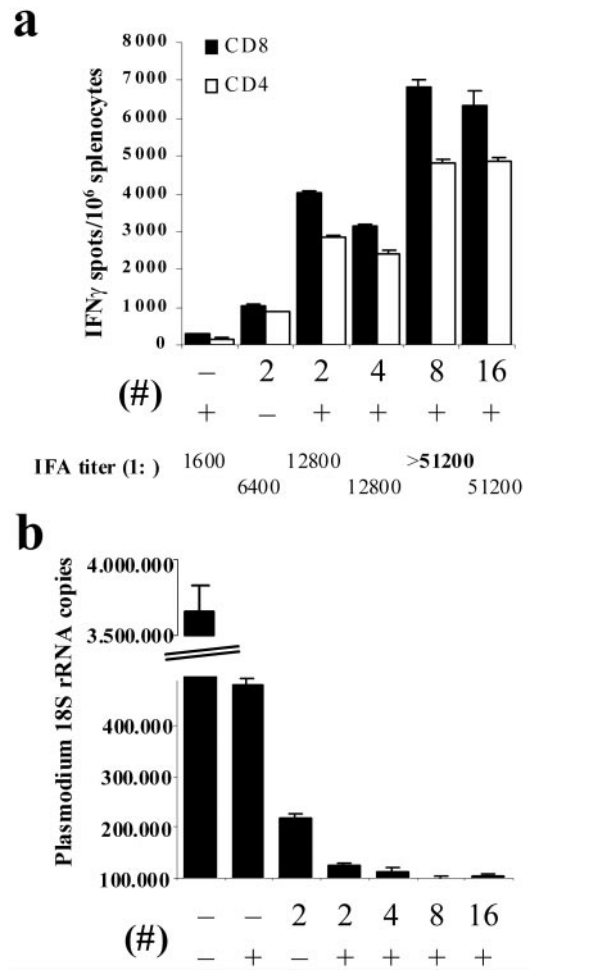

Fig. 2. (#) Effects of a booster given at varying time points (weeks after reAd priming) with 10^7 pfu VacPyCS (+) and controls (−). (*a*) CS-specific T cell responses and anti-sporozoite antibody titers and (*b*) inhibition of liver stage development after sporozoite inoculation. Data in *a* and *b* were obtained 2 weeks after booster and are each representative of four experiments.

for priming, the administration of a recombinant vaccinia virus expressing the same foreign antigen elicited a potent booster effect (6, 9, 16).

Because the initial immune response elicited by AdPyCS decreased with time, we decided to vary the timing of the VacPyCS booster to determine when it would provide maximal enhancement of the primary immune response.

The relative number of splenic CS-specific $CD8^+$ and $CD4^+$ T cells was highest when the booster was given 8 or more weeks after the initial immunizing dose of AdPyCS, rather than at shorter time intervals. Anti-sporozoite antibody titers followed the same pattern (Fig. 2*a*). They were highest when the mice were boosted 8 or 16 weeks after priming. The inhibition of liver stage development of the parasites was complete; i.e., no parasite 18S rRNA could be detected by real-time PCR in any of the mice when the booster was given 8 weeks after the first immunizing dose. At earlier or later times, 2, 4, or 16 weeks, the mice also displayed a very high degree of inhibition of development (>99.9%) of these parasite stages (Fig. 2*b*).

Table 1. Percentage of mice completely protected against sporozoite-induced infection when primed by reAd and boosted with reVV after a variable number of weeks

reAd	Interval, wk	reVV	Protected/ challenged	% Sterile immunity
+	2	+	3/5	60
+	8	+	10/10	100
+	16	+	3/5	60
+		−	0/5	0
−		+	0/5	0
−		−	0/5	0

All mice were challenged 2 weeks after booster with 75 sporozoites. The numbers are representative of three identical experiments, except for the data that represent booster 16 weeks after priming.

The observation that the booster was most effective 8 weeks after priming with AdPyCS was corroborated by the most critical assay, namely, challenge with viable sporozoites resulting in sterile immunity. In fact, no patent infection was detected in any of these VacPyCS boosted mice at this time point. When the same booster was given, either earlier at 2 weeks or later at 16 weeks after priming, the protection was significantly lower. Some of the mice in these groups were not completely protected and developed parasitemia after sporozoite challenge (Table 1).

The Time Elapsed Between Priming with AdPyCS and VacPyCS Booster Affects the Number of Activated Cells and Apoptosis Rates of CS-Specific $CD8^+$ T Cells Detected After Booster. Protection elicited in BALB/c mice (H-$2K^d$ haplotype) after immunization with either AdPyCS alone or VacPyCS alone has previously been demonstrated to be mainly mediated by $CD8^+$ T cells, in addition

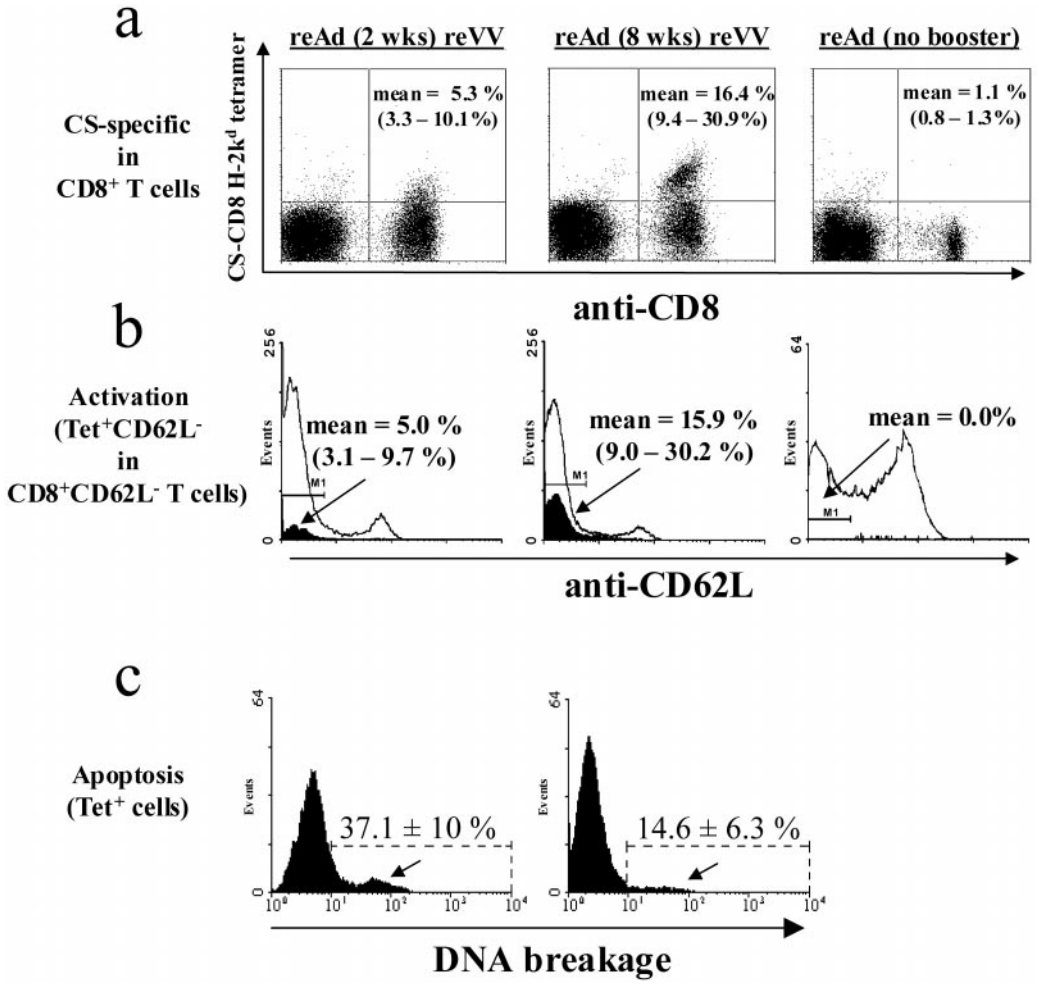

Fig. 3. Activation status and apoptosis rates of $CD8^+$ T cell populations obtained from the liver of mice immunized with reAd alone (control) or boosted with VacPyCS, 2 or 8 weeks after priming with AdPyCS. All data obtained 5 days after booster. Flow cytometry of lymphocytes showing (*a*) the percentage of CS-specific $CD8^+$ T cells, (*b*) the percentage of CS-specific (Tet^+) $CD8^+$ activated ($CD62L^-$) liver lymphocytes, and (*c*) the percentage of apoptosis of CS-specific (Tet^+) cells.

to antibodies, that recognize the epitope represented by the amino acid sequence SYVPSAEQI of the *P. yoelii* CS protein. Fluorescent complexes, generated with four $H\text{-}2K^d$ molecules bound to this epitope (Tetramers), and anti-CD8 antibodies can be used to detect these MHC class I-restricted CS-specific T cells.

First, we wanted to ascertain that the different numbers of IFN-γ-producing $CD8^+$ T cells observed by ELISPOT assays, when the VacPyCS booster was performed at different time intervals after AdPyCS priming, indeed corresponded to differences in the total number of $CD8^+$ T cells generated. Alternatively, other phenomena could result in less IFN-γ production, e.g., anergy of certain T cells or bias to a different cytokine-producing profile. Thus, lymphocytes were obtained 5 days after VacPyCS booster from the liver of several groups of mice primed/boosted at different time intervals, and stained with fluorescent CS-specific tetramers (Tet) together with a monoclonal anti-CD8 antibody. The flow cytometric analysis of these lymphocytes showed that the percentage of CS-specific $CD8^+$ T cells (Tet^+ $CD8^+$) were three times higher in the mice that received the VacPyCS booster 8 weeks after priming with AdPyCS, than in those boosted 2 weeks after priming. These specific $CD8^+$ T cells represented more than 30% of the total $CD8^+$ population in some of the mice (Fig. 3*a*). Moreover, the results of further analysis of this population by using the lymph node homing receptor L-selectin (CD62L), a surface molecule lost after lymphocyte activation, confirmed that the vast majority of these CS-specific $CD8^+$ T lymphocytes had an activated-cell phenotype (Tet^+ $CD8^+$ $CD62L^-$; Fig. 3*b*). These results confirmed that a VacPyCS booster administered 8 weeks after the AdPyCS priming induced more activated CS-specific protective $CD8^+$ T cells than the same booster given 2 weeks after priming.

We also observed an increase in the total number of activated $CD4^+$ cells generated after administration of the VacPyCS booster (data not shown). However, the numbers were not significantly different between the two prime/boost immunization regimens. We could not determine the number of CS-specific activated $CD4^+$ T cells because of the unavailability of MHC class II tetrameric complexes.

The large number of activated $CD8^+$ T cells, observed when the booster was administered 8 weeks after priming could in part be the result of decreased apoptosis of these cells compared with the one occurring when the booster was given after a shorter interval (2 weeks). In fact, it is known that greater cell death occurs when excess of antigen is presented to activated T cells in short intervals (17). We therefore compared the apoptosis levels of the CS-specific $CD8^+$ T cell populations obtained after administration of the VacPyCS booster to mice primed 2 and 8 weeks earlier with the AdPyCS. The levels of cell death after booster were more than two times greater for the CS-specific $CD8^+$ T cells (Tet^+ $CD8^+$) in mice boosted 2 weeks after priming than those boosted 8 weeks after priming (Fig. 3*c*).

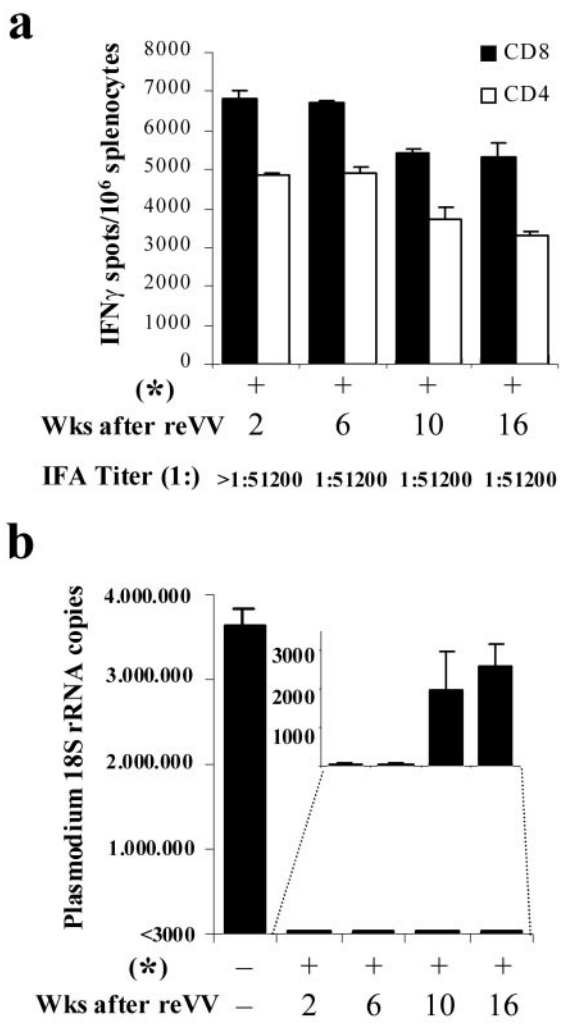

Fig. 4. (*) Persistence of immunity and inhibition of liver-stage development, at various time points (2, 6, 10, and 16 weeks) after recombinant vaccinia virus booster, in mice primed with reAd 8 weeks earlier. (*a*) Persistence of CS-specific $CD8^+$ and $CD4^+$ T cells and antibody titers. (*b*) Degree of inhibition of liver stage development.

Persistence of the Immune Responses, Including Protection. The anti-malaria immune responses induced by the priming/boosting regimen were maintained at high levels for a relatively long time, as demonstrated by determining the number of CS-specific $CD8^+$ and $CD4^+$ cells secreting IFN-γ by using the ELISPOT assay, as well as by measuring the IFA titers of antibodies against sporozoites in the sera of those mice (Fig. 4*a*). These results correlated with the inhibition of liver stage development, because parasite 18S rRNA was undetectable by the highly sensitive real-time PCR, whether the immunized mice were challenged at 2 or 6 weeks. They remained barely perceptible by this assay at 10 or even 16 weeks after the booster (Fig. 4*b*).

The level of protection, namely the failure to develop parasitemia after challenge with live sporozoites, at different times after boosting, is shown in Table 2. All of the mice challenged with VacPyCS developed complete protection against sporozoite challenge 2 and 6 weeks after booster, whereas all controls rapidly developed patent infections. Extensive protection was also observed 10 weeks after the booster, because 83% of the mice were completely protected. Moreover, an appreciable percentage of mice were still protected by week 16, i.e., four months after administration of the vaccinia vector.

Table 2. Percentage of mice protected against sporozoite challenge at 2, 6, 10, or 16 weeks after booster with reVV following priming with reAd 8 weeks earlier

reAd + reVV (8-week interval)	Weeks after reVV	Protected/ challenged	% Sterile immunity	Prepatent period, days
+	2	10/10	100	—
+	6	10/10	100	—
+	10	5/6	83	4.9
+	16	2/5	40	4.4
–	—	0/5	0	3.2

Discussion

A key question raised by our findings is to what degree they are applicable to the development of future vaccines, not only for malaria but also for other pathogens, particularly those with an intracellular stage, for which cell-mediated immunity is an important component of the protective immune response. This question might, at least partially, be answered in the future, by priming with another potent viral vector, such as Sindbis virus, which induces a strong primary CS-specific $CD8^+$ T cell response comparable to that induced by AdPyCS (18).

In the current experiments, it was not by chance that maximal protection occurred when the booster was administered 8 weeks after priming. This timing coincided precisely with a marked reduction of CS-specific $CD8^+$ and $CD4^+$ T cells elicited by priming with AdPyCS. In fact, we suspect that the strong cellular response to priming, originally done with 10^9 pfu/mouse of AdPyCS, might have been responsible for the very modest enhancement of the anti-malaria response to booster with VacPyCS. We found this surprising, because we had expected a much greater response. In the past, we had found that, when priming with a different vector, i.e., recombinant influenza virus, followed 2 weeks later by a booster with VacPyCS, there was a much greater enhancement of the immune response (6, 16). For this reason, we used 10^8 pfu of AdPyCS for priming in the current experiments, i.e., a 10-fold smaller viral dose. However, even by using this smaller dose, the primary immune response induced by AdPyCS (much stronger than that elicited by the recombinant influenza virus) was greatly enhanced only when the VacPyCS was administered 8 or more weeks later (Fig. 2).

The suboptimal boosting capacity of VacPyCS, observed when this vector was administered only 2 weeks after AdPyCS priming (Fig. 2) could be explained by the activation, as well as the apoptosis levels, of the $CD8^+$ T cells. In fact, we observed that, when the booster was given to mice after 2 weeks, the numbers of activated ($CD62L^-$) CS-specific $CD8^+$ T lymphocytes were considerably lower than at 8 weeks, suggesting an incomplete activation of this population. An additional reason could be that the percentage of apoptosis among both the $CD4^+$ and $CD8^+$ T cell populations, in mice boosted 2 weeks after priming, was 2 to 3 times higher than that after 8 weeks (Fig. 3*c*). The increased death of T cells after a second administration of the same antigen, when it is too close in time to the first administration, has been previously described as a protective and regulatory mechanism of the immune system, and is known as activation-induced cell death (AICD) (17).

Our results indicate that, not only are the number of CS-specific IFN-γ-secreting T cells after priming important, but even more so are the functional characteristics, such as the speed of response (19) and avidity changes of the TCR receptor (20), that determine the expansion and probably also the duration of the secondary immune response induced by the booster. In the present case, and probably in others, where priming generates large numbers of antigen-specific T cells, a substantial time interval has to pass between priming and boosting, to maximize

the expansion and effectiveness of the secondary responses, not only because of more precursors, but also because of greater efficacy of these cells, part of which by then would have become memory cells.

Whether the attenuated adenovirus type 5, from which a small segment of E3 and all E1 regions of the genome have been removed, still retains some immunomodulatory factors (21) that may have contributed to the level and persistence of priming and/or the maximal efficacy of boosting after 8 weeks remains to be determined.

The immunodominant CS protein and its epitopes, recognized by $CD8^+$ and $CD4^+$ T cells, respectively, as well as B cells, may also have contributed to the effective response induced by the AdPyCS-priming/VacPyCS-boosting regimen. However, this property is certainly not exclusive of this plasmodial antigen, because we also observed it after priming with influenza virus and boosting with a recombinant vaccinia virus expressing a cytotoxic T lymphocyte (CTL) epitope of the nucleoprotein of influenza (22).

VacPyCS, the viral vector we used for boosting the primary immune response, expresses the entire CS protein of *P. yoelii* (6) recognized by $CD8^+$ and $CD4^+$ and B cells. However, this recombinant vaccinia virus does not elicit good priming, most likely due to a strong immune response to its own numerous antigens. In sharp contrast, the same viral vector confers a potent booster to an earlier established primary immune response elicited either by a different microbial vector or some other form of presentation of the same antigen, sharing only the foreign sequence. It is also possible that some of the recombinant vaccinia virus-encoded proteins, such as cytokine or chemokine receptors IL-1β, IFN-γ, tumor necrosis factor (TNF), IL-18, and others (23), might modulate the primary response to the foreign antigen in a manner that results in an enhanced booster effect. These characteristics of the vector make VacPyCS an extremely potent booster, eliciting, as in the present experiments, not only very high levels of cell mediated immunity, but also very high titers of anti-sporozoite antibodies, leading to the generation (and selection) of highly effective foreign-antigen-specific T and B memory cells. Most important, it confers complete protection (sterile immunity) against sporozoite challenge. The long persistence of protection after the booster (>16 weeks) could be due to these same factors.

The concomitant very high levels of anti-sporozoite antibodies and parasite-specific T cells, the key components of two major mechanisms of protection, are of interest. It seems likely that this approach may also contribute to the prevention and/or treatment of other diseases, particularly those caused by pathogens that have an intracellular stage of development in host cells.

The efficacy of this prime/boost approach is supported by numerous data using other immunogens for priming and frequently a recombinant vaccinia virus expressing the same antigen for boosting. This viral vector enhances greatly the corresponding immune response to priming. Nevertheless, this is one of the first demonstrations, under well-defined conditions of immunization, of complete, long-lasting protection against an otherwise highly virulent parasite.

Whereas it is likely that other selected antigens or epitopes from the same or different stages of development will have to be included in the final vaccine formulation, we expect that this prime/boost approach will contribute to the development of effective vaccines, not only against malaria, but also against other diseases for which there are no satisfactory control measures.

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